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Optimization of phenoxazinone synthase production by response surface methodology and its application in Congo red decolourization

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Abstract

Background: Enzymatic decolourization has been recently proposed as a promising and eco-friendly method for treatment of synthetic dye-contaminated wastewaters. However, the processes require large quantities of enzymes, attracting significant attention in developing efficient methods for mass production of multifunctional enzymes. Several methods such as response surface methodology (RSM) and orthogonal experiment have been applied to optimize the parameters in bioprocesses for enzyme production.
Results: In the present study, a laccase-like enzyme, phenoxazinone synthase (PHS) originated from *Streptomyces antibioticus* was recombinantly expressed in *Escherichia coli* BL21 (DE3). The production of PHS in *E. coli* BL21 was optimized by response surface methodology based on Box-Behnken design. A full third-order polynomial model was generated by data analysis with Statistica 8.0 in which the optimal conditions for PHS production were calculated to be 1.525 mM $CuSO_4$ and 16.096 hrs induction at temperature of 29.88ºC. The highest PHS production under optimal conditions was calculated to be 4098.51 U/l using the established model. Average PHS production obtained from actual production processes carried out under the calculated optimal conditions was 4052.00 U/l, very close to the value predicted by the model. Crude PHS was subsequently tested in Congo red decolourization which exhibited a low decolourization rate of 27% without mediator. Several mediators were found to improve PHS-catalyzed Congo red decolourization, with the highest rate of 73.89% obtained with 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) as mediator under optimized conditions of 4000 U/l PHS activity, 10 μM ABTS, 100 μM Congo red, and 8 hrs reaction time.
Conclusion: Our results indicated that PHS recombinantly produced in *E. coli* BL21 was a prospective enzyme for decolorizing reactive dye Congo red.

Keywords: Congo red, decolourization, laccase, phenoxazinone synthase, response surface methodology (RSM).

INTRODUCTION

Synthetic dyes have gained increasingly broad applications in various industries in recent years, especially in countries with large manufacturing industries like China. The annual production of synthetic dyes is about 0.75 million tons in China, which accounted for 60% of world production in 2010. It was estimated that 10-20% of synthetic dyes were lost during production and application processes (Gouvea et al. 2000) generating billions of tons of wastewater every year globally. Nowadays, dye-contaminated wastewater has caused considerable environmental pollution, especially in developing countries (Zhang et al. 2012). Traditional technologies for wastewater treatment have been proved to be ineffective to treat wastewaters contaminated with various synthetic dyes. To solve

the problem, several specific treatments have been evaluated to remove synthetic dyes from wastewater (Forgacs et al. 2004) including adsorption (Namasivayam et al. 1994; Santhy and Selvapathy, 2006; Lin et al. 2010), chemical oxidation (Qiu et al. 2005), photocatalysis (Hachem et al. 2001; Kuo and Ho, 2001), biological decolourization (Pearcea et al. 2003; Selvam and Shanmuga Priya, 2012), and processes using combination treatment (Ghoreishi and Haghighi, 2003; Papić et al. 2004; Tantak and Chaudhari, 2006; Matveevich et al. 2009). However, the applications of these methods on a large scale were prohibited by high cost, limited applicability, and generation of toxic byproducts that were environmentally harmful. In comparison, enzymatic decolourization accomplishes total degradation of materials without producing toxic byproducts and has recently been evaluated as a promising and eco-friendly method for treatment of synthetic dye-contaminated wastewater (Chacko and Subramaniam, 2011).

Laccases (EC 1.10.3.2) are a family of copper-containing oxidases found in many plants, fungi, and microorganisms (Baldrian, 2006; Riva, 2006; Sharma et al. 2007) that catalyze the oxidation of substrates by molecular oxygen, which is concomitantly reduced to water. In recent years, laccases have been used in decolourization of dye-contaminated wastewater by catalyzing the oxidative degradation of a number of synthetic dyes (Wesenberg et al. 2003; Rodríguez Couto and Toca Herrera, 2006; Murugesan et al. 2007; Mishra et al. 2011). Studies reported so far indicated that bacterial laccases had higher activity and stability than fungal laccases and thus were more suitable for industrial applications (Singh et al. 2011). Phenoxazinone synthase (PHS), a bacterial oxidase originated from Streptomyces antibioticus, has been reported to exhibit high catalytic activity similar to that of laccases (Choy and Jones, 1981; Jones and Hopwood, 1984; Jones, 1985). In the present work, we aimed to develop and optimize recombinant PHS production in *Escherichia coli* BL21 (DE3) and study its potential application in Congo red decolourization.

A number of statistically designed experimental models have been applied to optimize the parameters in bioprocesses, among which the response surface methodology (RSM) is an effective experimental approach to investigate the optimum conditions in multivariable systems. Specifically, it has been used effectively to optimize culture parameters for many recombinant microorganisms (Lin et al. 2007; Zhao et al. 2008; Farliahati et al. 2010). In the present study, we used RSM to optimize the conditions for recombinant PHS production in *E. coli* BL21 and carried out preliminary evaluations of the produced PHS in decolourization of Congo red. Our results demonstrated that PHS produced from recombinant expression in *E. coli* BL21 was highly effective in decolorizing reactive dye Congo red and may have potential application in treatment of dye-contaminated wastewater.

MATERIALS AND METHODS

Chemicals and microorganism

Isopropyl β-D-1-thiogalactopyranoside (IPTG), Kanamycin sulphate, and Congo red were purchased from Merck, AMRESCO Inc., Tianjin Chemical Reagent Institute, Shanghai SSS Reagent Co. Ltd, and Shanghai Yuming Industrial Co. Ltd, respectively. 2,2'-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 1-hydroxybenzotriazole (HBT), 2,2',6,6'-tetramethylpiperidine-*N*-oxyl radical (TEMPO), copper sulphate ($CuSO_4$ x $5H_2O$), and sodium acetate were obtained from Sigma-Aldrich. All chemicals were of analytical grade.

Construction of recombinant *E. coli* BL21 (DE3) was carried out as previously described (Xie et al. 2011). A single colony picked from the slant culture was inoculated into 50 ml LB medium with 30 µg/ml Kanamycin in a 250 ml flask. The flask was incubated at 37ºC with shaking at 180 rpm for 12 hrs and the culture was used as standard inoculum for all fermentations after cultivation. Aliquots of 20 ml cell broth were centrifuged at 8000 rpm, 4ºC for 15 min. The cell pellets were combined, resuspended in LB medium, and sonicated at 220-240 W in ice bath for 10 min with 3 sec intervals. The sonicated suspension was subsequently centrifuged for 20 min at 8000 rpm, 4ºC to generate crude PHS extract in the supernatant.

Optimization of PHS production by response surface methodology via Box-Behnken design

The Box-Behnken design was used for the optimization of PHS production, with significant factors of time (X_1), temperature (X_2), and $CuSO_4$ concentration (X_3) being examined as critical variables. A two-way interaction quadratic model equation was employed to predict the optimal parameters:

$$y = k_0 + k_1X_1 + k_2X_2 + k_3X_3 + k_{11}X_1^2 + k_{22}X_2^2 + k_{33}X_3^2 + k_{12}X_1X_2 + k_{13}X_1X_3 + k_{23}X_2X_3 + k_{112}X_1^2X_2 + k_{122}X_1X_2^2 + k_{113}X_1^2X_3$$

where y was the response; k_0 was a constant; k_1, k_2, and k_3 were linear coefficients; k_{11}, k_{22}, and k_{33} were quadratic coefficients; k_{12}, k_{13}, k_{23}, k_{112}, k_{122}, and k_{113} were cross-coefficients.

A total of 15 experiments were carried out to estimate the coefficients in the equation. Experimental conditions and PHS yields in the 15 experiments are shown in Table 1. The experimental data were analyzed with Statistica 8.0 to generate optimal values of the factors. The statistical significance of the model was assessed by F-value and coefficient of determination, R^2.

Table 1. The Box-Behnken design of experiments and responses for PHS production.

Observations	Time (h)	Temperature (°C)	$CuSO_4$ (mM)	Response (U/l)
	X_1	X_2	X_3	y
1	12	24	1.5	1574
2	20	24	1.5	2388
3	12	36	1.5	787
4	20	36	1.5	651
5	12	30	1	1221
6	20	30	1	2252
7	12	30	2	2714
8	20	30	2	3989
9	16	24	1	2008
10	16	36	1	868
11	16	24	2	2714
12	16	36	2	923
13	16	30	1.5	3805
14	16	30	1.5	3795
15	16	30	1.5	3854

Congo red decolourization

Congo red decolourization experiments were performed in 50 ml flasks containing 20 ml reaction mixture of crude PHS and Congo red at various concentrations in 100 mM sodium acetate buffer (pH 6.0). The reaction mixture was incubated at 30ºC with shaking at 160 rpm. ABTS, HBT, and TEMPO were tested and compared as mediators to improve the efficiency of PHS-catalyzed decolourization. In addition, factors including reaction time, concentrations of mediator and Congo red, and PHS activity were investigated to obtain optimal conditions for PHS-catalyzed Congo red decolourization.

Analytical methods

PHS activity was determined spectrophotometrically by oxidation of substrate ABTS. The reaction was performed at 25ºC with 5 mM ABTS in 0.1 M sodium acetate buffer (pH 5.0) with the addition of a suitable amount of enzyme, and reaction progress was monitored by absorbance at 420 nm on UV-Vis spectrophotometer Genesys 10S from Thermo Scientific (USA). The unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidization of 1 µmol of ABTS per minute (ε_{420} = 36 mM^{-1} cm^{-1}). Congo red was monitored by absorbance at 500 nm on a UV spectrophotometer and quantified using a standard curve (R^2 = 0.9987). The yield of decolourization was calculated as follow:

$$y = \frac{M - m}{M} \times 100\%$$

where *M* was the amount of Congo red before decolourization and *m* was the amount of Congo red after decolourization.

RESULTS AND DISCUSSION

Box-Behnken design

The Box-Behnken design was applied to optimize laccase production from *Streptomyces psammoticus* in a recent publication (Niladeviet al. 2009). In the present study, we employed the Box-Behnken design to optimize recombinant PHS production from *E. coli* BL21. The experimental design and results are shown in Table 1. Regression data analysis by Statistica 8.0 (X_1, X_2, and X_3 represented time, temperature, and $CuSO_4$ concentration, respectively) generated a full third-order polynomial model for PHS production as shown below:

$$y = 1840.75 + 305.167X_1 + 364.313X_1^2 - 0.664.917X_2 + 882188X_2^2 + 60175X_3 + 225.188X_3^2 - 237.5X_1X_2 + 203.5X_1X_2^2 - 50.875X_1^2X_2 + 61X_1X_3 - 308.625X_1^2X_3 - 162.75X_2X_3$$

Three-dimensional response surfaces were generated to investigate interactions between the three factors tested, examine the combined effects of factors on PHS production, and determine optimal operation parameters for most efficient PHS production.

Response surface analysis is a powerful tool to interpret interactions in systems containing multiple variables, which are difficult to assess by conventional methods. The effects of interactions between operation parameters on PHS production were investigated with contour plots containing various possible combinations of two factors while keeping one factor constant at a time (Figure 1 and Figure 2).

The response surface plot of interactions between induction time and temperature on PHS production (Figure 1) showed that PHS production was directly proportional to induction time. Temperature was also found to have significant effect with favourable PHS production obtained at temperatures in the range of 22ºC to 3ºC. Taken together, the highest PHS production was achieved with 17 hrs induction at 30ºC.

A similar effect of interactions between $CuSO_4$ concentration and induction time on PHS production was observed as shown in Figure 3. PHS production initially increased with increasing $CuSO_4$ concentration and induction time, followed by a slight decrease at higher $CuSO_4$ concentration and extended induction time. According to the model generated as described above, maximal PHS production would be achieved with 16-17 hrs induction in the presence of 1.5 mM $CuSO_4$.

The effect of interactions between $CuSO_4$ concentration and temperature on PHS production is presented in Figure 2. PHS production initially increased with the enhancement of temperature and concentration of $CuSO_4$ in initial increasing $CuSO_4$ concentration and temperature, followed by a gradual decrease at higher $CuSO_4$ concentration and temperature. The highest PHS production was achieved at 30ºC with 1.5 mM $CuSO_4$.

The experimental results were fitted to a full third-order polynomial equation with multiple regression analysis. The calculated parameters and their significance levels are shown in Table 2.

The model adequacy was validated by coefficient R^2 of 0.9463, which indicated that the model could predict 94.63% of variability in the response. Significance of coefficients has been reported to be directly proportional to *P*-value. In the present work, the induction time (Q), temperature, and $CuSO_4$ concentration (L) were analyzed with *P*-values below 0.05, indicating that statistically significant high confidence level of about 95% was obtained.

The effects of induction time, temperature, and $CuSO_4$ concentration on PHS production were analyzed by response surface plots, which showed that all three variables had significant effects on PHS production. The response surfaces were analyzed with Statistica 8.0 to generate optimal values for the three factors which were determined to be 1.525 mM, 16.096 hrs, and 29.88ºC for $CuSO_4$ concentration, induction time, and temperature, respectively. The maximum PHS production was projected to be 4098.51 U/l under optimized conditions in the RSM model. In validation experiments carried out under optimized conditions projected by the model, an average PHS production of 4052.00 U/l, a value very close to that predicted by the model was obtained, verifying the adequacy and efficiency of our model.

Table 2. ANOVA results of RSM experiments for PHS production.

	Sum of square	*F*-value	*P*-value
Time (L)	1113032	4.98673	0.075869
Time (Q)	2224135	9.96482	0.025188[a]
Temperature (L)	3719628	16.66510	0.009518[a]
Temperature (Q)	10569013	47.35250	0.000992[a]
$CuSO_4$ (L)	1991010	8.92035	0.030563[a]
$CuSO_4$ (Q)	915247	4.10060	0.098740
1L*2L	225625	1.01087	0.360842
1L*3L	14884	0.06668	0.806519
2L*3L	105950	0.47469	0.521497

[a] $P < 0.05$.
R^2: 0.9463.

Decolourization of Congo red by PHS

Laccases produced from *Streptomyces* have been employed in previous studies to decolorize the recalcitrant dyes (Dubé et al. 2008; Niladevi et al. 2008) including Congo red, a dye widely used in textile dyeing and resistant to biodegradation treatment. To explore the potential application of PHS in dye degradation, we studied decolourization of Congo red by crude PHS extract and examined the effects of various factors on Congo red degradation. Congo red was quantified by absorbance at 500 nm and the yield of decolourization was calculated as described in Materials and Methods.

The effect of reaction time on yield of Congo red decolourization is illustrated in Figure 4. It was observed that the yield of decolourization increased only slightly from 24% to 27% within 1-10 hrs, indicating that crude PHS was unable to effectively decolorize Congo red. We believed that the observed inefficiency of crude PHS was likely attributed to the low redox potential of PHS from bacteria (around 400 mV), which was lower than that of PHS from fungi (around 800 mV) (Maté et al. 2011). Since small molecule mediators have been shown to improve decolourization rate in previous studies, we explored the effects of several such mediators on PHS-catalyzed decolourization of Congo red.

Effect of mediators on decolourization

During the catalytic oxidation of dyes, small molecule mediators facilitated electron transfer in which they were initially oxidized and then served as the oxidizing agents for dye molecules, leading to expedited dye degradation (Morozova et al. 2007). Specifically, several small molecules including HBT, TEMPO, and ABTS have been used as mediators in laccase-catalyzed dye degradation (Hu et al. 2009). In the present study, HBT, TEMPO, and ABTS were tested as mediators in PHS-catalyzed Congo red decolourization and the results are presented in Figure 5. We found that the best decolourization rate of 72.8% was achieved with ABTS (Figure 5c), much higher than those achieved with TEMPO (Figure 5a) and HBT (Figure 5b), which were below 55%.

Generally, ABTS were believed to exist in two forms in the redoxcouple of ABTS/$ABTS^{2+}$. In the reaction system, ABTS would be first oxidized to its cation radical ($ABTS^{+}$), then further oxidized to dication ($ABTS^{2+}$). The redox potential were found to be 472 mV for ABTS/$ABTS^{+}$ and 885 mV for $ABTS^{+}$/$ABTS^{2+}$, respectively (Bourbonnais et al. 1998). ABTS and $ABTS^{2+}$ would exist in a stable state during the reaction process with reversible electron transfer. In the present work, ABTS facilitated a decolourization rate of 72.8%, much higher than that obtained without mediator or with either HBT or TEMPO as mediator and was hence selected to be used in subsequent experiments for the study of factors affecting PHS-catalyzed Congo red decolourization.

Factors affecting Congo red decolourization

In the present study, the effect of each factor on Congo red decolourization was investigated in experiments in which a single factor was varied while other factors were kept constant.

The effect of ABTS concentration on decolourization of Congo red is shown in Figure 5c. Congo red decolourization rate initially increased with ABTS concentration and reached a maximum rate of 72.8% at 10 μM ABTS, and then decreased slightly at higher ABTS concentration. We speculated that ABTS at low concentrations acted as a facilitator of electron transfer to accelerate PHS-catalyzed decolourization of Congo red. However, ABTS, as a substrate for PHS, also competed with Congo red for PHS binding when present at higher concentrations in solution, resulting in reduced yield of Congo red decolourization. Accordingly 10 μM ABTS was used in further experiments in Congo red decolourization.

The effect of PHS activity on decolourization of Congo red is shown in Figure 6a. The decolourization rate showed fast increase with PHS activity in the range of 800 to 4000 U/l, followed by a slower increase at higher enzyme activities, indicating that the decolourization approached maximal reaction rate. We considered the optimal PHS activity to be 4000 U/l for Congo red decolourization.

We also tested the decolourization rate at varying Congo red concentrations from 30 to 140 μM in the presence of 10 μM ABTS. As shown in Figure 6b, the decolourization rate steadily increased to the maximum point of 72.8% with Congo red concentration going up to 100 μM, and then quickly decreased at higher Congo red concentrations, which might be attributed to structure changes in PHS. In the presence of low concentrations of Congo red, active centres of PHS might be in full contact with dye molecules, which promoted high reaction activities. However, in the presence of higher concentrations of Congo red, dye molecules would occupy most active centres in PHS, inducing structural changes, which may lead to enzyme inactivation and decreased decolourization rate (Ramsay and Nguyen, 2002). The optimum Congo red concentration for decolourization was observed to be 100 μM in our study.

Finally, the effect of reaction time on the yield of decolourization in the presence of 10 μM ABTS is illustrated in Figure 6c. It was found that the decolourization rate of Congo red gradually increased to 73% within 10 hrs, indicating that crude PHS with ABTS as a mediator was able to decolorize Congo red effectively. Specifically, the decolourization rate increased to 72.8% within 8 hrs before approaching plateau, indicating that reaction time of 8 hrs was optimal for the decolourization process.

In summary, the optimal conditions identified for Congo red decolourization were 100 μM Congo red, 10 μM ABTS, 4000U/l crude PHS, and 8 hrs reaction time. Validation experiments were carried out under the identified optimal conditions from which an average decolourization rate of 73.89% was obtained, a dramatic increase from the 39% decolourization rate achieved with laccases from *Streptomyces coelicolor* (Dubé et al. 2008). Our results should promote exploration of industrial application of PHS in treatment of dye-contaminated wastewater.

CONCLUDING REMARKS

The recombinant production of PHS in *E. coli* BL21 (DE3) was optimized by response surface methodology via Box-Behnken design. A full third-order polynomial model for PHS production was established with a determination coefficient of 0.9463 using data analysis by Statistica 8.0. The highest PHS activity was calculated to be 4098.51 U/l under optimal conditions of 1.525 mM $CuSO_4$ and 16.096 hrs induction at 29.88ºC. PHS productions were carried out under calculated optimal conditions and an average PHS activity of 4052.00 U/l was obtained which was very close to the value predicted by the model.

We then tested the crude PHS in decolourization of reactive dye Congo red and found that PHS without mediator was inefficient in catalyzing Congo red decolourization as shown in a low decolourization rate of 27%. However, addition of mediator ABTS significantly boosted the decolourization reaction with optimal rate of 73.89% achieved at optimal conditions of 4000 U/l PHS activity, 10 μM ABTS, 100 μM Congo red, and 8 hrs reaction time. Our findings should promote exploration of industrial application of PHS in treatment of dye-contaminated wastewater.

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REFERENCES

BALDRIAN, P. (2006). Fungal laccases - occurrence and properties. *FEMS Microbiology Reviews*, vol. 30, no. 2, p. 215-242. **[CrossRef]**

BOURBONNAIS, R.; LEECH, D. and PAICE, M.G. (1998). Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. *Biochimica et Biophysica (BBA) - Acta-General Subjects*, vol. 1379, no. 3, p. 381-390. **[CrossRef]**

CHACKO, J.T. and SUBRAMANIAM, K. (2011). Enzymatic degradation of azo dyes - A review. *International Journal of Environmental Sciences*, vol. 1, no. 6, p. 1250-1260.

CHOY, H.A. and JONES, G.H. (1981). Phenoxazinone synthase from *Streptomyces antibiotics*: Purification of the large and small enzyme forms. *Archives of Biochemistry and Biophysics*, vol. 211, no. 1, p. 55-65. **[CrossRef]**

DUBÉ, E.; SHARECK, F.; HURTUBISE, Y.; BEAUREGARD, M. and DANEAULT, C. (2008). Decolourization of recalcitrant dyes with a laccase from *Streptomyces coelicolor* under alkaline conditions. *Journal of Industrial Microbiology & Biotechnology*, vol. 35, no. 10, p. 1123-1129. **[CrossRef]**

FARLIAHATI, M.R.; RAMANAN, R.N.; MOHAMAD, R.; PUSPANINGSIH, N.N.T. and ARIFF, A.B. (2010). Enhanced production of xylanase by recombinant *Escherichia coli* DH5α through optimization of medium composition using response surface methodology. *Annals of Microbiology*, vol. 60, no. 2, p. 279-285. **[CrossRef]**

FORGACS, E.; CSERHÁTI, T. and OROS, G. (2004). Removal of synthetic dyes from wastewaters: A review. *Environment International*, vol. 30, no. 7, p. 953-971. **[CrossRef]**

GHOREISHI, S.M. and HAGHIGHI, R. (2003). Chemical catalytic reaction and biological oxidation for treatment of non-biodegradable textile effluent. *Chemical Engineering Journal*, vol. 95, no. 1-3, p. 163-169. **[CrossRef]**

GOUVEA, C.A.K.; WYPYCH, F.; MORAES, S.G.; DURÁN, N.; NAGATA, N. and PERALTA-ZAMORA, P. (2000). Semiconductor-assisted photocatalytic degradation of reactive dyes in aqueous solution. *Chemosphere*, vol. 40, no. 4, p. 433-440. **[CrossRef]**

HACHEM, C.; BOCQUILLON, F.; ZAHRAA, O. and BOUCHY, M. (2001). Decolourization of textile industry wastewater by the photocatalytic degradation process. *Dyes and Pigments*, vol. 49, no. 2, p. 117-125. **[CrossRef]**

HU, M.R.; CHAO, Y.P.; ZHANG, G.Q.; XUE, Z.Q. and QIAN, S.J. (2009). Laccase-mediator system in the decolorization of different types of recalcitrant dyes. *Journal of Industrial Microbiology & Biotechnology*, vol. 36, no. 1, p. 45-51. **[CrossRef]**

JONES, G.H. and HOPWOOD, D.A. (1984). Molecular cloning and expression of the phenoxazinone synthase gene from *Streptomyces antibioticus*. *Journal of Biological Chemistry*, vol. 259, no. 22, p. 14151-14157.

JONES, G.H. (1985). Regulation of phenoxazinone synthase expression in *Streptomyces antibioticus*. *Journal of Bacteriology*, vol. 163, no. 3, p. 1215-1221.

KUO, W.S. and HO, P.H. (2001). Solar photocatalytic decolorization of methylene blue in water. *Chemosphere*, vol. 45, no. 1, p. 77-83. **[CrossRef]**

LIN, S.S.; DOU, W.F.; XU, H.Y.; LI, H.Z.; XU, Z.H. and MA, Y.H. (2007). Optimization of medium composition for the production of alkaline β-mannanase by alkaliphilic *Bacillus* sp. N16-5 using response surface methodology. *Applied Microbiology and Biotechnology*, vol. 75, no. 5, p. 1015-1022. **[CrossRef]**

LIN, K.S.; CHENG, H.W.; CHEN, W.R. and WU, C.F. (2010). Synthesis, characterization, and adsorption kinetics of titania nanotubes for basic dye wastewater treatment. *Adsorption - Journal of the International Adsorption Society*, vol. 16, no. 1-2, p. 47-56. **[CrossRef]**

MATÉ, D.; GARCÍA-RUIZ, E.; CAMARERO, S. and ALCALDE, M. (2011). Directed evolution of fungal laccases. *Current Genomics*, vol. 12, no. 2, p. 113-122. **[CrossRef]**

MATVEEVICH, V.A.; DUKA, G.G.; GONTSA, M.V.; YAMBARTSEV, V.F. and MOKANU, L.V. (2009). Removal of direct dyes from textile wastewater by means of combined methods. *Surface Engineering and Applied Electrochemistry*, vol. 45, no. 4, p. 312-317. **[CrossRef]**

MISHRA, A.; KUMAR, S. and PANDEY, A.K. (2011). Laccase production and simultaneous decolorization of synthetic dyes in unique inexpensive medium by new isolates of white rot fungus. *International Biodeterioration & Biodegradation*, vol. 65, no. 3, p. 487-493. **[CrossRef]**

MOROZOVA, O.V.; SHUMAKOVICH, G.P.; SHLEEV, S.V. and YAROPOLOV, Y.I. (2007). Laccase-mediator systems and their applications: A review. *Applied Biochemistry and Microbiology*, vol. 43, no. 5, p. 523-535. **[CrossRef]**

MURUGESAN, K.; DHAMIJA, A.; NAM, I.H.; KIM, Y.M. and CHANG, Y.S. (2007). Decolourization of reactive black 5 by laccase: Optimization by response surface methodology. *Dyes and Pigments*, vol. 75, no. 1, p. 176-184. **[CrossRef]**

NAMASIVAYAM, C.; JEYAKUMAR, R. and YAMUNA, R.T. (1994). Dye removal from wastewater by adsorption on "waste" Fe(III)/Cr(III) hydroxide. *Waste Management*, vol. 14, no. 7, p. 643-648. **[CrossRef]**

NILADEVI, K.N.; SHEEJADEVI, P.S. and PREMA, P. (2008). Strategies for enhancing laccase yield from *Streptomyces psammoticus* and its role in mediator-based decolorization of azo dyes. *Applied Biochemistry and Biotechnology*, vol. 151, no. 1, p. 9-19. **[CrossRef]**

NILADEVI, K.N.; SUKUMARAN, R.K.; JACOB, N.; ANISHA, G.S. and PREMA, P. (2009). Optimization of laccase production from a novel strain - *Streptomyces psammoticus* using response surface methodology. *Microbiological Research*, vol. 164, no. 1, p. 105-113. **[CrossRef]**

PAPIĆ, S.; KOPRIVANAC, N.; LONCARIĆ BOZIĆ, A. and METES, A. (2004). Removal of some reactive dyes from synthetic wastewater by combined Al(III) coagulation/carbon adsorption process. *Dyes and Pigments*, vol. 62, no. 3, p. 291-298. **[CrossRef]**

PEARCE, C.I.; LLOYD, J.R. and GUTHRIE, J.T. (2003). The removal of colour from textile wastewater using whole bacterial cells: A review. *Dyes and Pigments*, vol. 58, no. 3, p. 179-196. **[CrossRef]**

QIU, Z.; HE, Y.; LIU, X. and YU, S. (2005). Catalytic oxidation of the dye wastewater with hydrogen peroxide. *Chemical Engineering and Processing*: *Process Intensification,* vol. 4, no. 9, p. 1013-1017. **[CrossRef]**

RAMSAY, J.A. and NGUYEN, T. (2002). Decoloration of textile dyes by *Trametes versicolor* and its effect on dye toxicity. *Biotechnology Letters*, vol. 24, no. 21, p. 1757-1761. **[CrossRef]**

RIVA, S. (2006). Laccases: Blue enzymes for green chemistry. *Trends in Biotechnology*, vol. 24, no. 5, p. 219-226. **[CrossRef]**

RODRÍGUEZ COUTO, S. and TOCA HERRERA, J.L. (2006). Industrial and biotechnological applications of laccases: A review. *Biotechnology Advances*, vol. 24, no. 5, p. 500-513. **[CrossRef]**

SANTHY, K. and SELVAPATHY, P. (2006). Removal of reactive dyes from wastewater by adsorption on coir pith activated carbon. *Bioresource Technology*, vol. 97, no. 11, p. 1329-1336. **[CrossRef]**

SELVAM, K. and SHANMUGA PRIYA, M. (2012). Biological treatment of Azo dyes and textile industry effluent by newly isolated White rot fungi *Schizophyllum commune* and *Lenzites eximia*. *International Journal of Environmental Sciences*, vol. 2, no. 4, p. 1926-1935.

SHARMA, P.; GOEL, R. and CAPALASH, N. (2007). Bacterial laccases. *World Journal of Microbiology & Biotechnology*, vol. 23, no. 6, p. 823-832. **[CrossRef]**

SINGH, G.; BHALLA, A.; KAUR, P.; CAPALASH, N. and SHARMA, P. (2011). Laccase from prokaryotes: A new source for an old enzyme. *Reviews in Environmental Science and Bio/technology*, vol. 10, no. 4, p. 309-326. **[CrossRef]**

TANTAK, N.P. and CHAUDHARI, S. (2006). Degradation of azo dyes by sequential Fenton's oxidation and aerobic biological treatment. *Journal of Hazardous Materials*, vol. 136, no. 3, p. 698-705. **[CrossRef]**

WESENBERG, D.; KYRIAKIDES, I. and AGATHOS, S.N. (2003). White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnology Advances*, vol. 22, no. 1-2, p. 181-187. **[CrossRef]**

XIE, Y.C.; JIA, H.H.; XIE, B.S.; ZHU, Q.H.; JIA, L.S. and WEI, P. (2011). Optimization of the expression of phenoxazinone synthase from *Streptomyces antibioticus*. *Biotechnology Bulletin*, vol. 41, p. 176-180.

ZHANG, L.; SUN, Y.; GUO, D.; WU, Z. and JIANG, D. (2012). Molecular diversity of bacterial community of dye wastewater in an anaerobic sequencing batch reactor. *African Journal of Microbiology Research*, vol. 6, no. 35, p. 6444-6453. **[CrossRef]**

ZHAO, J.; WANG, Y.; CHU, J.; ZHANG, S.; ZHUANG, Y. and YUAN, Z. (2008). Statistical optimization of medium for the production of pyruvate oxidase by the recombinant *Escherichia coli*. *Journal of Industrial Microbiology & Biotechnology*, vol. 35, no. 4, p. 257-262. **[CrossRef]**

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Figures

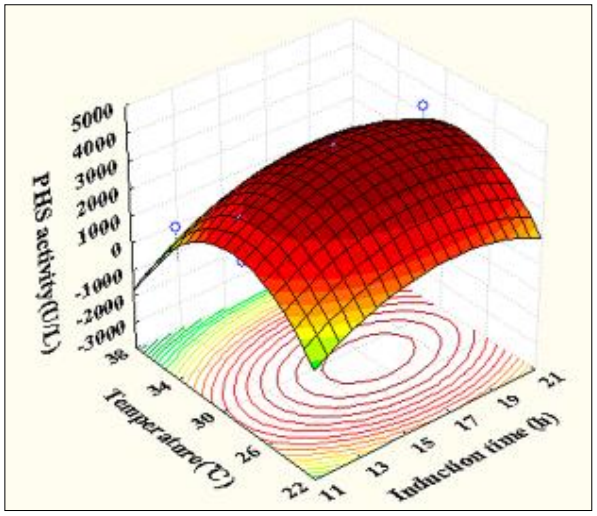

Fig. 1 RSM figure and contour of time and temperature on PHS production.

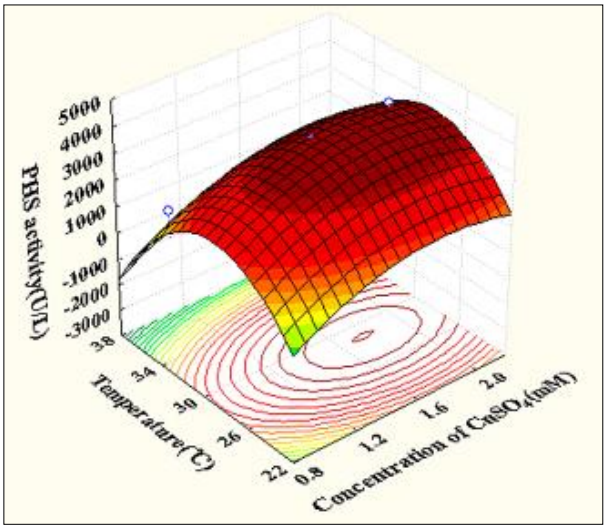

Fig. 2 RSM figure and contour of temperature and Cu^{2+} concentration on PHS production.

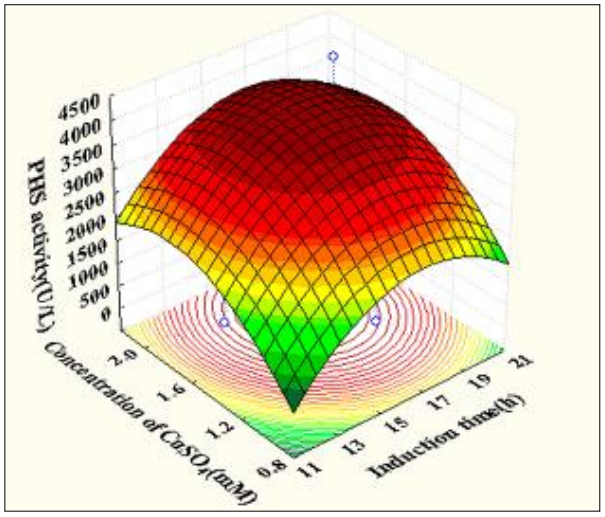

Fig. 3 RSM figure and contour of time and Cu^{2+} concentration on PHS production.

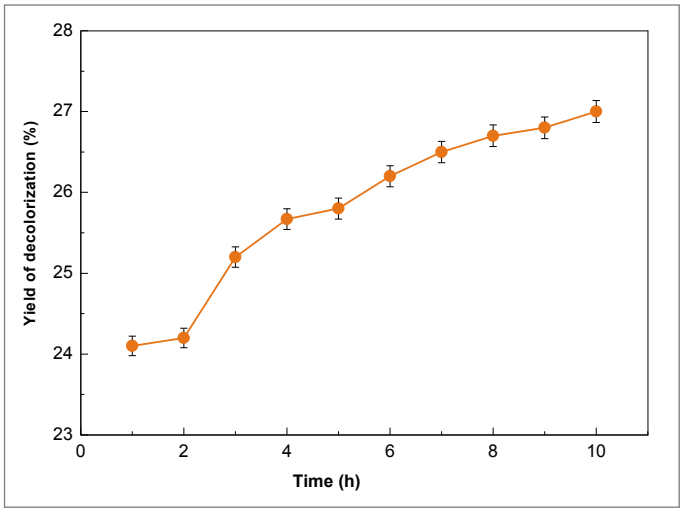

Fig. 4 Decolourization of Congo red by PHS without mediator.

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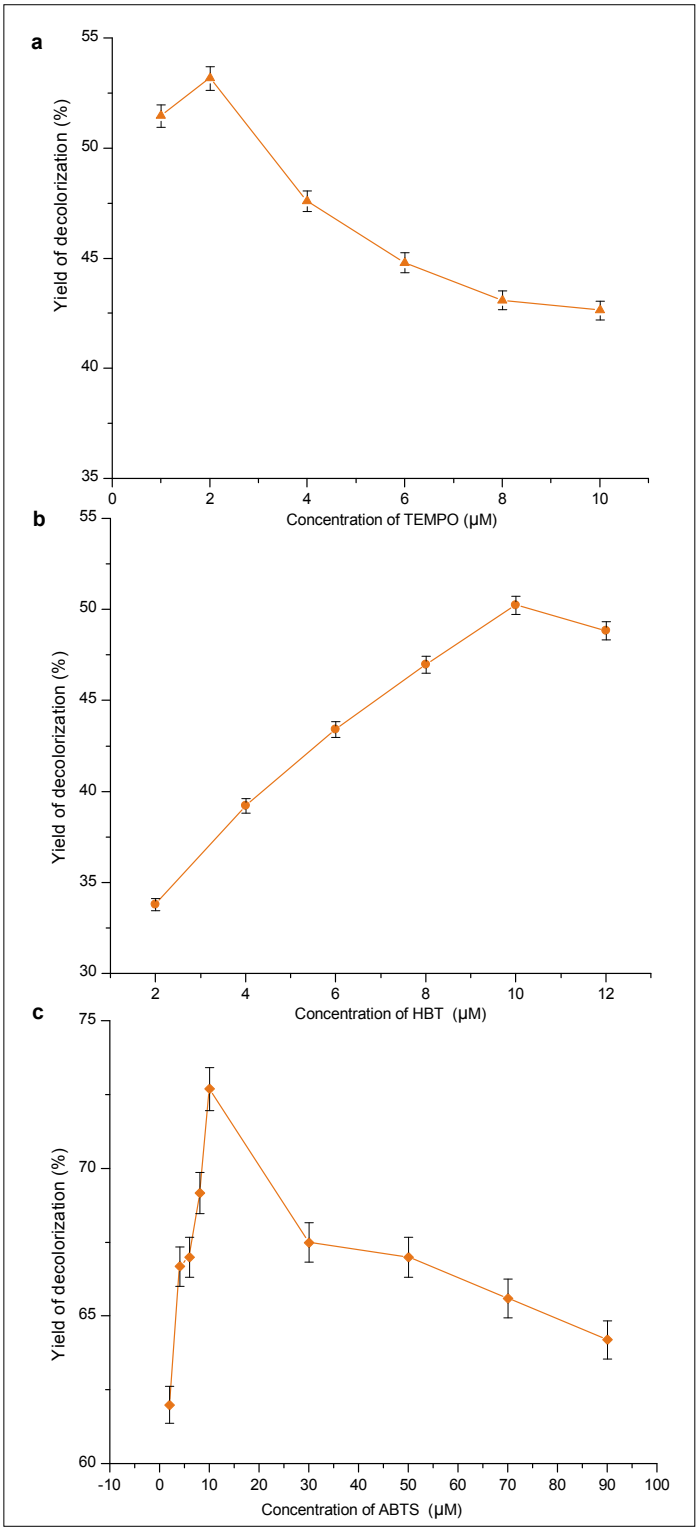

Fig. 5 Congo red decolourization by PHS with mediators (a) TEMPO; (b) HBT; (c) ABTS.

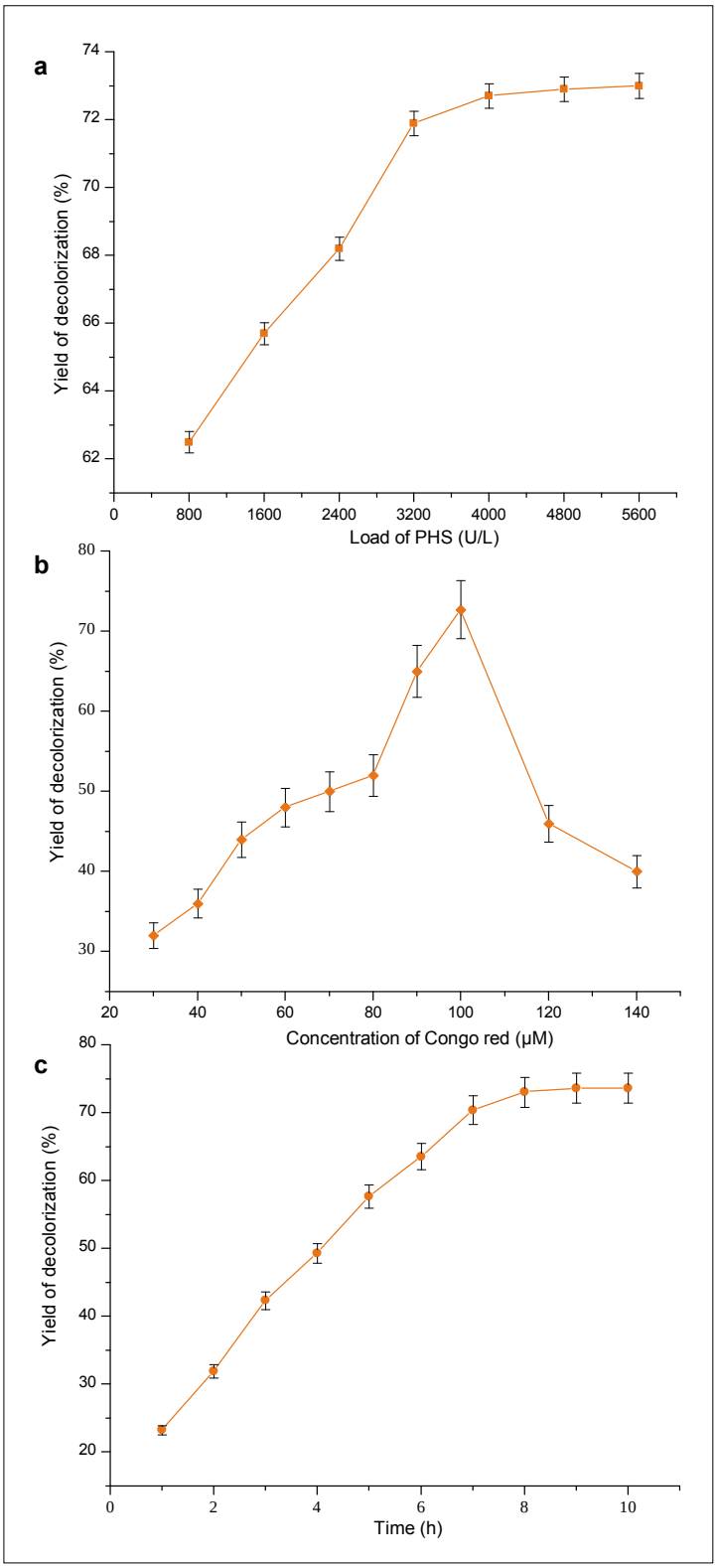

Fig. 6 (a) Effect of PHS activity on Congo red decolourization (10 μM ABTS, 100 μM Congo red, 8 hrs reaction time); (b) Effect of Congo red concentration on decolourization (10 μM ABTS, 4000 U/l PHS activity, 8 hrs reaction time); (c) Effect of reaction time on Congo red decolourization (10 μM ABTS, 4000 U/l PHS activity, 100 μM Congo red).

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